DOCA sensitive pendrin expression in kidney, heart, lung and thyroid tissues

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Abstract: BACKGROUND/AIMS: Pendrin (SLC26A4), a transporter accomplishing anion exchange, is expressed in inner ear, thyroid gland, kidneys, lung, liver and heart. Loss or reduction of function mutations of SLC26A4 underlie Pendred syndrome, a disorder invariably leading to hearing loss with enlarged vestibular aqueducts and in some patients to hypothyroidism and goiter. Renal pendrin expression is up-regulated by mineralocorticoids such as aldosterone or deoxycorticosterone (DOCA). Little is known about the impact of mineralocorticoids on pendrin expression in extrarenal tissues. METHODS: The present study utilized RT-qPCR and Western blotting to quantify the transcript levels and protein abundance of Slc26a4 in murine kidney, thyroid, heart and lung prior to and following subcutaneous administration of 100 mg/kg DOCA. RESULTS: Slc26a4 transcript levels as compared to Gapdh transcript levels were significantly increased by DOCA treatment in kidney, heart, lung and thyroid. Accordingly pendrin protein expression was again significantly increased by DOCA treatment in kidney, heart, lung and thyroid. CONCLUSION: The observations reveal mineralocorticoid sensitivity of pendrin expression in kidney, heart, thyroid and lung

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Original Paper

DOCA Sensitive Pendrin Expression in Kidney, Heart, Lung and Thyroid Tissues

Lisann Pelzl[a] Tatsiana Pakladok[a] Ganesh Pathare[a] Hajar Fakhri[a] Diana Michael[a] Carsten A. Wagner[b] Markus Paulmichl[c] Florian Lang[a]

[a]Department of Physiology, University of Tuebingen; [b]Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich; [c]Institute of Pharmacology and Toxicology, Paracelsus Medical University, Salzburg

Key Words
Slc26a4 • Acidosis • Alkalosis • Liver • Kidney

Abstract
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Introduction

Pendrin is an electroneutral anion exchanger transporting chloride, bicarbonate, iodide and further anions [1-3]. Loss or reduction of function mutations in the pendrin gene

Florian Lang
Department of Physiology, University of Tübingen
Gmelinstr. 5, 72076 Tübingen (Germany)
Tel. +49 7071 29-72194, Fax +49 7071 29-5618
E-Mail florian.lang@uni-tuebingen.de

(SLC26A4) [4-9] lead to autosomal-recessive Pendred syndrome (PDS) with sensorineural hearing loss paralleled by enlarged vestibular aqueducts [3, 10, 11]. Lack of functional pendrin may further result in an iodide organification defect with an enhanced risk of developing goiter and hypothyroidism [3, 12, 13]. The development of goiter and hypothyroidism in Pendred syndrome is variable and may depend on nutritional iodide intake [13, 14].

SLC26A4 is expressed in a variety of tissues including thyroid gland, inner ear, kidney, lung, liver and heart [12, 14-17]. SLC26A4 mediated transport is critically important for proper development of the inner ear [18, 19]. The precise contribution of SLC26A4 to iodide transport in thyroid glands has, however, been a matter of debate [12, 14, 20, 21]. SLC26A4 contributes to cell volume regulation [22], airway transport regulation [23, 24] as well as HCO_3^- secretion and Cl^- reabsorption in the distal nephron [17, 25-28]. Renal tubular SLC26A4 influences expression and activity of the epithelial Na^+ channel ENaC and therefore impacts on blood pressure regulation [28-33].

SLC26A4 expression and function is up-regulated by ambient pH, aldosterone, intestinal natriuretic hormone, angiotensin II and the pro-inflammatory cytokines, interleukin (IL)-4 and IL-13. [28, 30, 34-39].

Mineralocorticoid sensitivity of renal SLC26A4 expression is well established [28, 30, 32]. Mineralocorticoid receptors are, however, expressed in a wide variety of further tissues [40], including colon, lung, cardiac myocytes, blood vessels, hippocampus, adipose tissue and thyroids [41-46]. Mineralocorticoids play a decisive role in a wide variety of functions, such as renal and colonic Na^+ and K^+ transport [45], salt appetite [47], hypertension [48], cardiac remodelling and fibrosis [49-52], stiff endothelial cell syndrome (SECS) [53-56], vascular stiffness [57] and calcification [58, 59], as well as apoptosis in hippocampal neurons [60]. Accordingly, aldosterone influences the expression of a wide variety of genes related to those functions [58, 61-66].

Little is known about the effect of aldosterone on SLC26A4 expression in tissues other than kidney, such as heart, lung and thyroid gland. The present study thus explored the effect of the mineralocorticoid deoxycorticosterone (DOCA) on the transcript levels and protein abundance of Slc26a4 in murine kidney, cardiac, lung and thyroid tissues.

Materials and Methods

Animals

Experiments were performed in 8-10 week old female and male wild type mice . All animal experiments were conducted according to German and Swiss laws for the welfare of animals and were approved by local authorities. The animals had free access to food (C1310, Altromin, Heidenau, Germany) and tap water. Where indicated the animals were treated with subcutaneous injections of deoxycorticosterone (DOCA , Sigma, Taufkirchen, Germany) 3 hours prior to determination of Slc26a4 transcript and protein levels.

RT-PCR analysis

To determine Slc26a4 mRNA abundance in mouse organs total RNA was extracted from both tissues using Trifast Reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo$(dT)_{12-18}$ primers (Invitrogen, Karlsruhe, Germany) and SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). cDNA samples were treated with RNase H (Invitrogen, Karlsruhe, Germany). Quantitative RT-PCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) and iTaq™ Sybr Green Supermix with ROX (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The following primers were used (5'→3' orientation): Slc26a4 s: TTCGGTCTCTACTCTGCCTTT; Slc26a4 as: CCCACCATTAAACTGACCACG; Gapdh s: AGGTCGGTGTGAACGGATTTG; Gapdh as: TGTAGACCATGTAGTTGAGGTCA. The specificity of the PCR products was confirmed by analysis of the melting curves and in addition by agarose gel electrophoresis. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the 68 °C method using Gapdh as an internal reference.

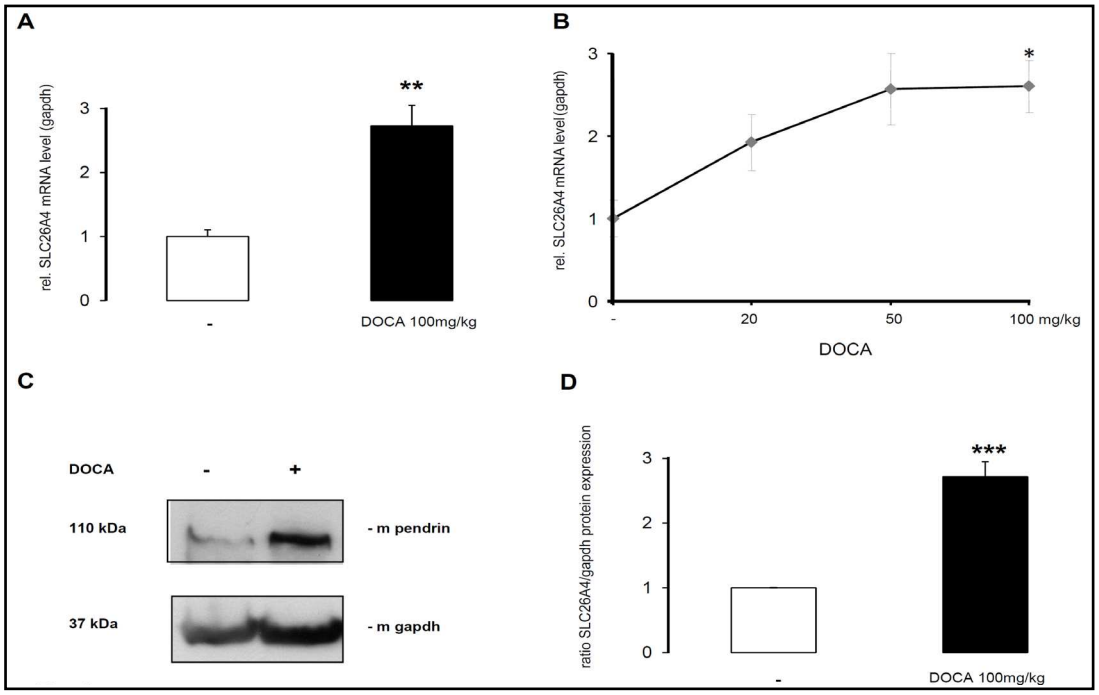

Fig. 1. Slc26a4 mRNA and protein abundance in kidney without and with DOCA treatment. A. Arithmetic means ± SEM (n = 5) of Slc26a4 mRNA abundance in kidney from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). **($p<0.01$) indicates statistically significant difference with respect to untreated animals. B. Dose response curve of DOCA induced up-regulation of Slc26a4 mRNA levels. *($p<0.05$) indicates statistically significant difference to untreated animals. C. Representative original blot for pendrin protein abundance in kidney from animals without treatment (-DOCA) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (+DOCA). D. Arithmetic means ± SEM (n = 5) of Slc26a4 protein abundance in kidney from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). ***($p<0.001$) indicates statistically significant difference to untreated animals.

Membrane preparation and western blot analysis

For determination of Slc26a4 protein abundance, tissue samples were homogenized in an ice-cold K-HEPES buffer (200 mM mannitol, 80 mM HEPES, 41 mM KOH, pH 7.5) containing a protease inhibitor mix (Complete Mini, Roche Diagnostics, Germany; 1 tablet in a volume of 10 ml). Samples were centrifuged at 1500xg for 10 min at 4°C. Subsequently, the supernatant was transferred to a new tube and centrifuged at 12000xg for 1 h at 4°C. The resultant pellet was resuspended in K-HEPES buffer containing protease inhibitors. After measurement of the total protein concentration (Bio-Rad D_c Protein Assay; Bio-Rad, Hercules, CA, USA), 100 µg of crude membrane proteins were solubilized in Laemmli sample buffer, and SDS-PAGE was performed on 8% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). After blocking with 5% milk powder in Tris-buffered saline/0.1% Tween-20 for 60 min, the blots were incubated with the respective primary antibodies (rabbit anti-pendrin 1:1000 [67] and rabbit monoclonal anti-gapdh antibody (37 kDa; Cell Signaling Technology) 1:2000, diluted in 1% milk/TBS-T) either for 2 h at room temperature or overnight at 4°C. After washing and subsequent blocking, the membranes were incubated for 1 h at room temperature with the secondary antibody conjugated with horse radish peroxidase (HRP) (1:2000, Cell Signaling). After washing antibody binding was detected with the ECL detection reagent (Amersham). All Bands were analyzed with Quantity One Software (Biorad).

Statistical analysis

As indicated, data are provided as means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed t-test where applicable. Only differences with $p<0.05$ were considered statistically significant.

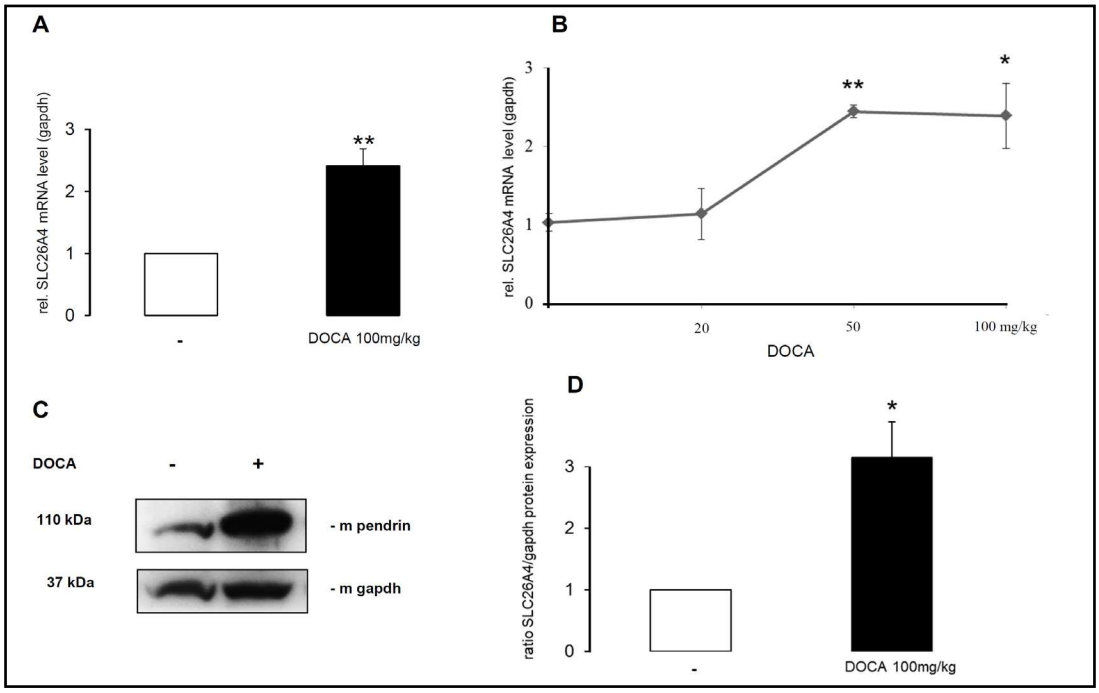

Fig. 2. Slc26a4 mRNA and protein abundance in heart without and with DOCA treatment. A. Arithmetic means ± SEM (n = 9) of Slc26a4 mRNA abundance in heart from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). **(p<0.01) indicates statistically significant difference to untreated animals. B. Dose response curve of DOCA induced up-regulation of Slc26a4 mRNA levels. *(p<0.05), **(p<0.01) indicates statistically significant difference to untreated animals. C. Representative original blot for pendrin protein abundance in heart from animals without treatment (-DOCA) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (+DOCA). D. Arithmetic means ± SEM (n = 5) of SLC26A4 protein abundance in heart from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). *(p<0.05) indicates statistically significant difference to untreated animals.

Results

Semi-quantitative reverse transcription polymerase chain reaction (RT-qPCR) was employed to quantify the transcript levels encoding Slc26a4 and Western blotting was utilized to quantify the Slc26a4 protein abundance in murine kidney, thyroid, heart and lung prior to and following subcutaneous administration of 20, 50 or 100 mg/kg DOCA.

As illustrated in Fig. 1A,B, the abundance of Slc26a4 mRNA in the kidney was significantly increased following treatment of mice with the deoxycorticosterone DOCA (100 mg/kg). Normalization of the Slc26a4 transcript levels to the transcript levels of the house keeping gene Gapdh yielded the Slc26a4/Gapdh transcript level ratio, which was significantly increased by DOCA treatment (by 160%, n = 5). The increase of Slc26a4 transcript levels following DOCA treatment was paralleled by an increase of Slc26a4 protein abundance (Fig. 1C and Fig. 1D).

Similar to what was observed in the kidney, DOCA (50 or 100 mg/kg) treatment significantly increased Slc26a4 transcript levels in the heart (Fig. 2A,B). The increase of the cardiac Slc26a4 transcript levels following DOCA treatment was similarly evidenced by an increase of the Slc26a4/Gapdh transcript level ratio in the heart (by 160%, n = 9). The increase of cardiac Slc26a4 transcript levels following DOCA treatment was similarly paralleled by an increase of cardiac SLC26A4 protein abundance (Fig. 2C and Fig. 2D).

As illustrated in Fig. 3A,B, both, Slc26a4 mRNA and protein were expressed in the lung. Similar to what was observed in kidney and heart, the mineralocorticoid treatment (DOCA 50 or 100 mg/kg) significantly increased lung Slc26a4 transcript levels. The increase of

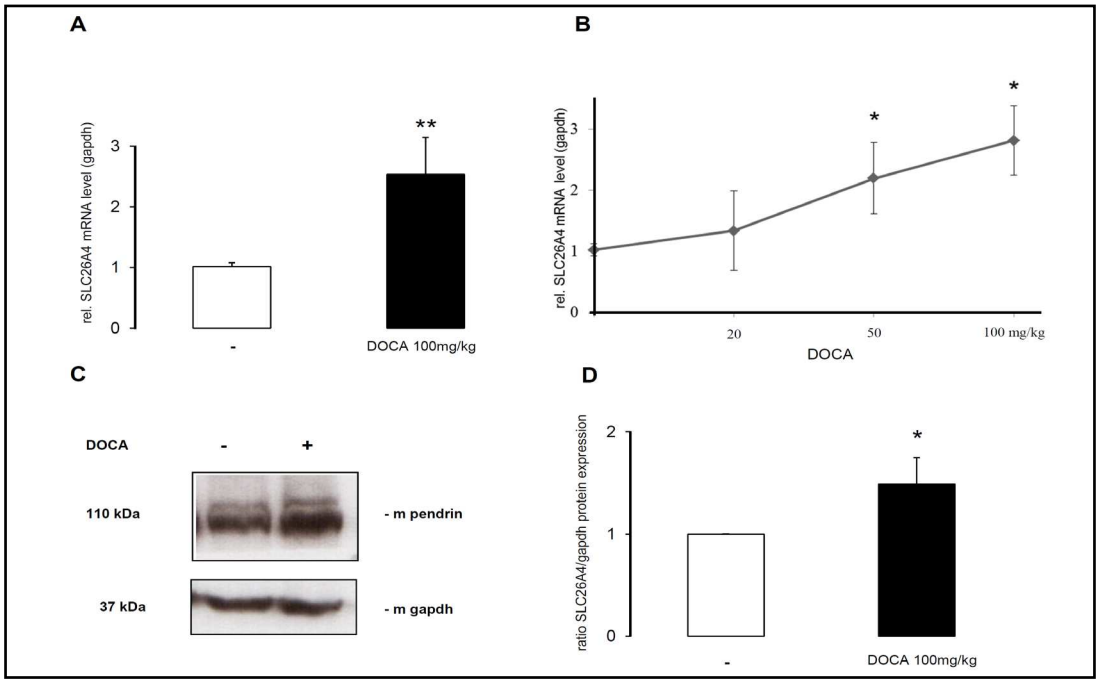

Fig. 3. SLC26A4 mRNA and protein abundance in lung without and with DOCA treatment. A. Arithmetic means ± SEM (n = 9) of Slc26a4 mRNA abundance in lung from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). **(p<0.01) indicates statistically significant difference to untreated animals. B. Dose response curve of DOCA induced up-regulation of Slc26a4 mRNA levels. *(p<0.05) indicates a statistically significant difference to untreated animals. C. Representative original blot for pendrin protein abundance in lung from animals without treatment (-DOCA) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (+DOCA). D. Arithmetic means ± SEM (n =6) of SLC26A4 protein abundance in lung from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). *(p<0.05) indicates statistically significant difference to untreated animals.

Slc26a4 transcript levels following DOCA treatment was again evidenced by an increase of Slc26a4/Gapdh transcript level ratio (by 153%, n = 9). The increase of Slc26a4 transcript levels following DOCA treatment was again paralleled by an increase of Slc26a4 protein abundance (Fig. 3C and Fig. 3D).

Lastly, DOCA (50 or 100 mg/kg) treatment increased Slc26a4 transcript levels in thyroid gland (Fig. 4A,B). The increase of the thyroid Slc26a4 transcript levels following DOCA treatment was again evidenced by an increase of the Slc26a4/Gapdh transcript level ratio (by 319%, n = 7). The increase of thyroid Slc26a4 transcript levels following DOCA treatment was again paralleled by an increase of thyroid Slc26a4 protein abundance (Fig. 4C and Fig. 4D).

Discussion

The present study demonstrates that pendrin transcript (Slc26a4) levels and pendrin protein abundance in kidney, heart, lung and thyroids are modified by the mineralocorticoid deoxycorticosterone (DOCA).

The present study did not attempt to define the molecular mechanisms involved in the up-regulation of the carrier. A candidate signaling molecule is the serum & glucocorticoid inducible kinase SGK1, which is strongly upregulated by mineralocorticoids and is a powerful regulator of a variety of channels and transporters [68]. SGK1 is partially effective by up-regulating the transcription factor NFκB [69], which contributes to the stimulating effect of

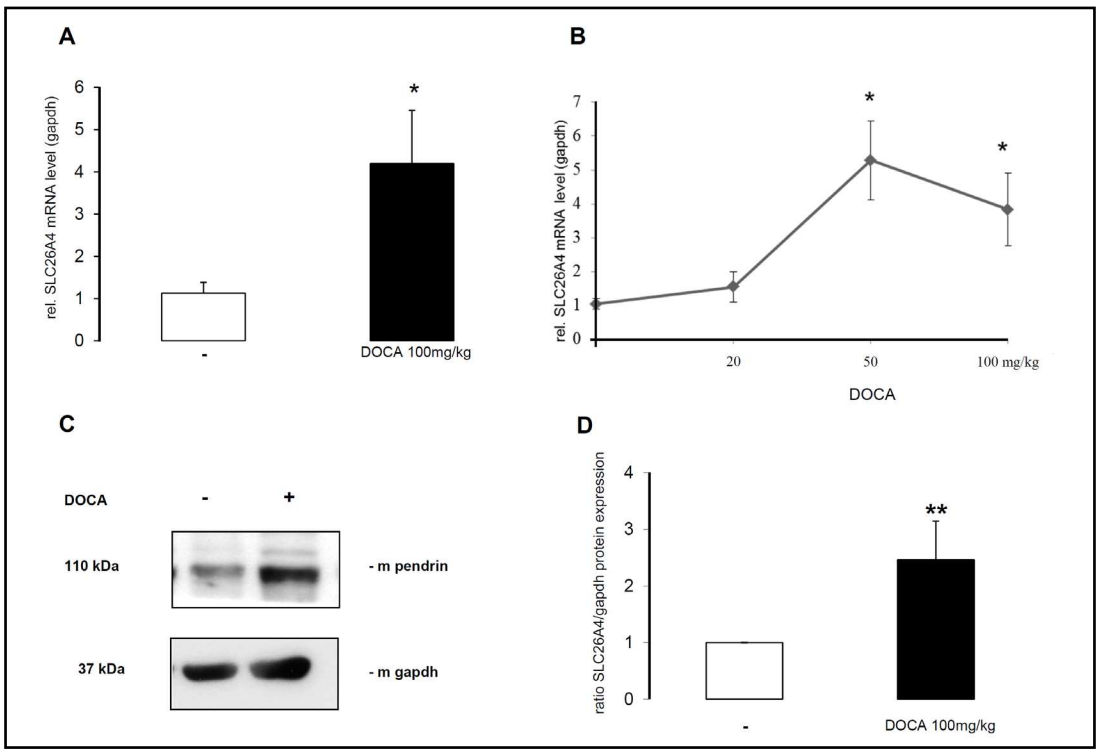

Fig. 4. SLC26A4 mRNA and protein abundance in thyroid gland without and with DOCA treatment. A. Arithmetic means ± SEM (n = 7) of Slc26a4 mRNA abundance in thyroid gland from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). *($p<0.05$) indicates statistically significant difference to untreated animals. B. Dose response curve of DOCA induced up-regulation of Slc26a4 mRNA levels. *($p<0.05$) indicates statistically significant difference to untreated animals. C. Representative original blot for pendrin protein abundance in thyroid gland from animals without treatment (-DOCA) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (+DOCA). D. Arithmetic means ± SEM (n = 5) of SLC26A4 protein abundance in thyroid gland from animals without treatment (white bar) and 3 hours following subcutaneous injection of 100 mg/kg DOCA (black bar). **($p<0.01$) indicates statistically significant difference to untreated animals.

mineralocorticoids on inflammation and fibrosis [70]. Whether or not NFκB is involved in the up-regulation of SLC26A4 expression during mineralocorticoid excess or inflammation, remains to be tested.

The effect of DOCA on SLC26A4 expression in the kidney is expected to affect HCO_3^- secretion and Cl^- reabsorption across the distal nephron [17, 25-28]. Moreover, SLC26A4 is expected to modify expression and function of the renal epithelial Na^+ channel ENaC resulting in enhanced renal tubular NaCl transport and increased blood pressure [28-33]. As a matter of fact, pendrin deficient mice are resistant to aldosterone-induced hypertension [32].

The up-regulation of SLC26A4 expression in lung tissue may serve to foster transport of anions across the aiway epithelium [23, 24]. Notably, SLC26A4 expression is up-regulated in bronchial asthma and chronic obstructive pulmonary disease. The carrier presumably does play an active role in respiratory inflammation and tissue destruction/remodeling [23, 24].

The effect of DOCA treatment on SLC26A4 protein abundance is only moderate in lung tissue. Nevertheless, the effect is statistically significant. Possibly, mineralocorticoids up-regulate SLC26A4 protein abundance only in a subset of cells in lung tissue. If so, Western blotting of whole organ tissue would underestimate the effect on mineralocorticoid sensitive cells.

The functional role of pendrin sensitivity to DOCA in other tissues is less obvious. In theory, the up-regulation of pendrin in the thyroid could impact on the formation of thyroid

hormones. Loss of function SLC26A4 mutations, however, do not necessarily affect iodide transport and hormone release in thyroid glands [12, 14, 20, 21]. Thus, aldosterone sensitive regulation of SLC26A4 in the thyroid may be relevant for functions other than thyroid hormone release. It is worth mentioning, however, that the heart has been claimed to possess all enzymes required for thyroid hormone formation [16].

SLC26A4 is further known to serve cell volume regulation [22]. Parallel activation of Na^+/H^+ exchangers and Cl^-/HCO_3^- exchangers participate in cell volume increase [71, 72], as they accomplish cellular NaCl uptake, which in turn is followed by osmotically driven water entry. Extrusion of H^+ by Na^+/H^+ exchange and extrusion of HCO_3^- by Cl^-/HCO_3^- exchange, respectively, are osmotically not relevant, as H^+ and HCO_3^- are replenished in the cell from CO_2 via H_2CO_3 [10, 71]. Along those lines, aldosterone is known to upregulate Na^+/H^+ exchange in the kidney [73-80], heart [81-85], and a variety of other extrarenal tissues [86-103].

Opposite regulation of SLC26A4 activity and expression have previously been observed in liver and kidney following alterations of acid base balance [15]. Following acidosis Slc26a4 transcript levels, protein abundance and/or activity are down-regulated in kidney [15, 27, 67, 104] and Slc26a4 transcript levels are up-regulated in liver [15]. Similarly, carbonic anhydrase inhibition or deficiency downregulate Slc26a4 expression in the kidney [15, 105, 106] but up-regulate Slc26a4 transcript levels in liver [15]. Conversely, bicarbonate induced metabolic alkalosis up-regulates Slc26a4 expression in the kidney [15, 105], but down-regulates Slc26a4 transcript levels in liver [15]. The opposite regulation of pendrin in liver and kidney may serve the complimentary functions of these organs in the regulation of systemic acid-base balance [107]. As mineralocorticoids stimulate renal tubular H^+ secretion and thus cause alkalosis [27], their effect on renal pendrin may similarly aim to influence acid base balance.

In conclusion, Slc26a4 transcripts and protein were observed in kidney, thyroids, lung and heart. Moreover, SLC26A4 abundance was sensitive to DOCA not only in kidney, but as well in heart, thyroids and lung. The present observation point to DOCA sensitive pendrin functions beyond its well established role in inner ear, thyroids and kidney.

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